REMARKS

Claims 1, 4-9 and 12-16 are pending and stand rejected. The Examiner has rejected Claims 1 and 4-8 as indefinite pursuant to 35 U.S.C. 112, second paragraph. The Examiner has rejected all of the claims as not enabled pursuant to 35 U.S.C. 112, first paragraph. In addition, the Examiner has raised double patenting rejections for certain claims. All art rejections have been withdrawn.

A. Formal Rejections

The Examiner rejects Claims 1 and 4-8 under 35 U.S.C. 112 as allegedly indefinite. The term "genomic" allegedly creates ambiguity. The Examiner indicates language would remedy the alleged defect if the insertion is clearly indicated to be "into the plant virus nucleic acid that encodes a coat protein." Without acquiescing to the rejection, but to further the prosecution, the claims have been amended in the manner suggested by the Examiner. The right to prosecute the unamended claims (or similar claims) in the future is expressly hereby reserved.

B. The Claims are enabled

The Examiner has rejected all of the claims as not enabled pursuant to 35 U.S.C. 112, first paragraph. Applicants disagree. To be enabling, the specification of the patent must teach those skilled in the art how to make and use the full scope of the claimed invention without undue experimentation. Undue experimentation is analyzed by consideration of eight factors set forth in *In re Wands*. The *Wands* factors are:

> (1) the quantity of experimentation necessary, (2) the amount of direction or guidance presented, (3) the presence or absence of working examples, (4) the nature of the invention, (5) the state of the prior art, (6) the relative skill of those in the art, (7) the predictability or unpredictability of the art, and (8) the breadth of the claims.

In re Wands, 858 F.2d 731, 737 (Fed. Cir. 1988).

Looking first at the breadth of the claims, the above-made amendments to Claim 1 make it clear that insertion is into the plant virus nucleic acid that encodes a coat protein. Thus, the claims are limited to insertion at a specific place that is shown to be operable. Importantly, the claims are further limited to a particular embodiment such that "said inserted nucleotide

sequence . . . [is] *an addition* to the existing native functional plant viral nucleic acid." (see Claims 1 and 9, emphasis added). Thus, the question of whether particular sequences of the native coat protein can be removed does not enter into the picture. The Examiner is requested to take note of the statement: "The use of addition inserts appears to offer more flexibility than replacement inserts in some instances." (specification, page 5).[1]

There are working examples – not just one example. Indeed, "[t]o demonstrate the wide applicability of this invention, antigenic sites of three different animal viruses were used." (see specification, page 7). The three different foreign epitopes used are FMDV, HRV and HIV. In spite of the different nature and size of these inserts, all are shown to be operable.

With respect to guidance, it is broadly taught to use "an exposed part of the coat protein of the virus." (specification, page 3).[2] It is preferred that "a particularly valuable group of viruses" are used "as vectors are those in which the nucleic acid coding for the capsid is a separate moiety from that which codes for other functional molecules and whose coat proteins have a B-barrel structure." (see specification, page 4). In addition, those viruses "which share structural similarities but whose coat proteins do not have a B-barrel structure may also be modified in accordance with this invention . . ." (see specification, page 5, emphasis added). Where the coat proteins have a B-barrel structure, it is suggested that the "loops between the individual strands" of the beta-sheet be utilized. (see specification, page 4). In this regard, the loops of different viruses are discussed and compared:

> "One of the principal differences between the comoviruses and picornaviruses is that the protein subunits of comoviruses lack the large insertions between the strands of the B-barrels found in picornaviruses though the fundamental architecture of the particles is very similar. The four loops (. . . see Figure 2) between the B-sheets are not critical for maintaining the tructural integrity of the virions but, in accordance with this invention, are used as sites of expression of foreign peptide sequences . . ."

(see specification, page 11). Thus, other viruses and other loops can be utilized.

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[1] The Examiner's citations to Takamatsu *et al.* and Usha *et al.* support this point – and therefore do not support the Examiner's rejection.

[2] The Examiner's argument that "no substantial relevant guidance is provided in regard to insertion into other nucleic acid regions of CPMV that encode other proteins . . ." is completely inappropriate, given the present claims. Applicants are required only to enable to scope of the present claims, which specify insertion into the coat protein – not other proteins.

In addition, there is guidance as to the "size of the foreign peptide" insert (see specification, page 2).[3] Specifically, the specification teaches peptides with "more than 5 amino acids" and demonstrate with working examples that between 19 and 24 amino acid inserts are functional (see examples).[4] Without acquiescing to the Examiner's rejection, but to further the prosecution, the claims have been amended to include this size limitation. The right to prosecute the unamended claims (or similar claims) in the future is expressly hereby reserved.

As to the quantity of experimentation needed, the skill in the art, and predictability, the Examiner is asked to take note of the publication by Uhde *et al.* (submitted herewith). The publication starts out by noting the importance of "the presentation of foreign epitopes on the surface of viral particles" – citing several publications (references 23, 26, 27 and 28) by one of the present inventors (Lomonossoff). Uhde et al. apply this technology to *Potato virus X* by putting epitopes in the coat protein (e.g. fusing epitopes to the N-terminus end as shown in Figure 1 of the publication), including putting two epitopes in tandem.

The importance of the Uhde *et a*l. publication is, first and foremost, that it provides actual evidence – not mere speculation – that the technology can be applied to other viruses without undue experimentation. Second, it shows that it is not necessary to limit the placement of inserts to loops; attachment to the N-terminus works nicely. Third, it points out that CPMV is not the easiest system to work with and that other viruses, like the *Potato virus X*, lack "the size and packaging constraints of icosahedral viruses like CPMV." (See the Uhde *et al* publication, page 337). This latter point negates the attempt by the Examiner to apply the concerns of Porta *et al.* to all viral systems. This is not a case where the present applicants worked with the easiest system and seek application to more difficult systems. This is a case where the applicants exemplified the technology in a relatively difficult system (having "size and packaging constraints") but where the technology can be applied readily to more robust systems.

In sum, those skilled in the art are taught by the present specification, in a preferred embodiment, to a) utilize the coat protein, b) add the insert without replacing native sequence,

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[3] The Examiner is simply mistaken when asserting that "No discussion or guidance is provided as to unpredictability of results that can arise depending on the size . . of a particular insert." (Office Action, page 8).

[4] These are not limiting. Indeed, the specification teaches that "[d]etermination of the permissible and most appropriate size . . . maybe achieved in each particular case by experiment in the light of the present disclosure." (specification, page 5)

and c) limit the size of the insert – all of which (i.e. when this quidance is combined) limit the amount of experimentation and make success predictable. Because each of these features is found in the claims as amended, the enablement rejection must be withdrawn.

C. Double Patenting

The Examiner has rejected Claims 9 and 12-16 under the judicially created doctrine of double patenting over claims 1-9 of U.S. Patent 5,874,087 stating that the claims are not patentably distinct over the claims of the '087. The Examiner has also rejected Claims 9 and 12-16 under the judicially created doctrine of double patenting over claims 22-28 of U.S. Patent 5,958,422. Provided Applicants' claims are otherwise found allowable, Applicants may split out these claims into a separate application with the required Terminal Disclaimer. This would permit Claims 1 and 4-8 to issue. The Examiner is requested to call the undersigned prior to another Office Action in order to discuss this procedure.

CONCLUSION

The Applicants believe that the arguments and claim amendments set forth above traverse the Examiner's rejections and, therefore, request that these grounds for rejection be withdrawn for the reasons set above. Should the Examiner believe that a telephone interview would aid in the prosecution of this application, the Applicants encourage the Examiner to call the undersigned collect at 617-984-0616.

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Expression of multiple foreign epitopes presented as synthetic antigens on the surface of *Potato virus X* particles

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Summary. We describe the construction of recombinant *Potato virus X* (PVX) vectors expressing two different epitopes, ep4 and ep6, from *Beet necrotic yellow vein virus* (BNYVV). The seven-amino-acid epitopes were expressed as N-terminal coat protein fusions and were displayed on the surface of PVX particles. Particle assembly into full virions was successful even though no wild type coat protein subunits were present, and the epitopes could be detected in crude extracts and purified virus preparations with appropriate antibodies. A construct containing both epitope sequences in tandem was also prepared. The resulting PVX particles could be detected by antibodies against ep4 and ep6, either individually or simultaneously, showing that both epitopes were accessible. In addition mixed infections with PVX vectors containing the individual ep4 and ep6 sequences were carried out. This resulted in the formation of PVX particles displaying ep4 alone, ep6 alone, or both epitopes. These experiments demonstrate for the first time that PVX can be utilized to present multiple epitopes, either tandemly on every coat protein subunit or as heteromultimeric assemblies, both of which could be useful vaccination strategies. The production of epitope-presenting viruses in which every coat protein subunit contains a foreign epitope allows the high-level expression of defined numbers of foreign antigen sites, making such viruses useful standards for immune detection.

Introduction

Viruses are widely used as vectors for the transient expression of foreign proteins in plants [27]. While the production of full-length proteins is often the objective of such expression experiments, another important application is the presentation of

foreign epitopes on the surface of viral particles [23]. After fusing the appropriate coding sequence to a highly expressed structural gene, such as a coat protein (CP) gene, transient expression of foreign proteins or peptides can be achieved at very high levels. The foreign coding region may be inserted so that the heterologous peptide is added to the end of the coat protein or located within an internal loop, since these regions tend to be displayed on the surface of the viral particle. As long as the inserted peptide is not too large, then neither the replication nor the assembly of the recombinant virus is impaired.

Epitope-presentation systems have been developed based on several different plant viruses, but the most popular are *Cowpea mosaic virus* (CPMV) [7, 26, 37] and *Tobacco mosaic virus* (TMV) [33, 35] since their structures have been solved at atomic resolution by X-ray crystallography. More recently, *Potato virus X* (PVX) has been investigated as a possible presentation system [9, 24, 25, 32] although much of the structural information available for this virus has been inferred rather than determined empirically. It is assumed that the N-terminal part of the CP is presented on the particle surface, so this is the ideal position to insert heterologous peptides [2, 18].

PVX, the type species of the genus *Potexvirus*, infects many solanaceous species [19]. The virus normally spreads by mechanical contact, and artificial infection with PVX is achieved by mechanical inoculation of host plants such as *Nicotiana benthamiana*, which become systemically infected. The RNA genome has five open reading frames (ORFs), the most 3′ of which is controlled by a subgenomic promoter and encodes the 236-residue coat protein. PVX has been used as an expression vector for full-length proteins by incorporating an additional subgenomic promoter [8, 16]. It has also been widely used to study virus induced gene silencing [4, 10].

The major advantage of flexuous filament particles such as PVX for epitope presentation is that the capsid has no intrinsic size or packaging constraints, and many copies of the target epitope can potentially be displayed on the particle surface. The first experiment describing protein-presentation on PVX particles involved an N-terminal CP fusion with green fluorescent protein (GFP) [9]. Experimental investigations showed that proteins from 8.5 to 31 kD in size were only expressed on the intact PVX particle surface if wild type coat protein was also present. This was achieved by the insertion of a ribosomal skip site, the 2A peptide of *Foot-and-mouth disease virus* (FMDV) [11, 12, 30], between the heterologous sequence and the CP sequence [9, 30]. More recently, PVX has been used to express coat protein fusions with a single-chain variable fragment (scFv) antibody [32], a rotavirus major capsid protein (VP6) [25] and an epitope from glycoprotein 41 of *Human immunodeficiency virus* (HIV) [24]. In the present study we demonstrate that PVX can be used to display two synthetic antigens derived from BNYVV, either alone or in combination. We propose that synthetic antigens can be used as a standard reference for immune detection. Because of their particular structure, such antigens should also be useful as immunogens for vaccination.

Materials and methods

Expression constructs

The GFP-CP fusion vector CXI (a kind gift from S. Santa-Cruz, Horticulture Research International (HRI), East Malling, United Kingdom) contains a GFP-2A-CP gene fusion under the transcriptional control of a subgenomic CP promoter (Fig. 1) [9]. This was modified by replacing the 2A and GFP sequences with BNYVV sequences, resulting in the expression of BNYVV epitopes as 5′-end in-frame CP fusions (Fig. 1). BNYVV epitope sequences were amplified using primers that incorporated restriction sites suitable for cloning into CXI (Table 1). Part of the 5′-end of the PVX CP was also amplified using primers incorporating an *Eag*I restriction site and an additional coding sequence for the respective foreign BNYVV epitopes (Table 1). Following amplification, the PCR products were cloned into the pCR2.1 vector (Invitrogen), released using restriction enzymes *Eag*I and *Spe*I, and inserted into the CXI vector, which had been prepared using the same enzymes. This produced recombinant vectors pPVX^{ep4}CP, pPVX^{ep6}CP and pPVX^{ep4ep6}CP, respectively

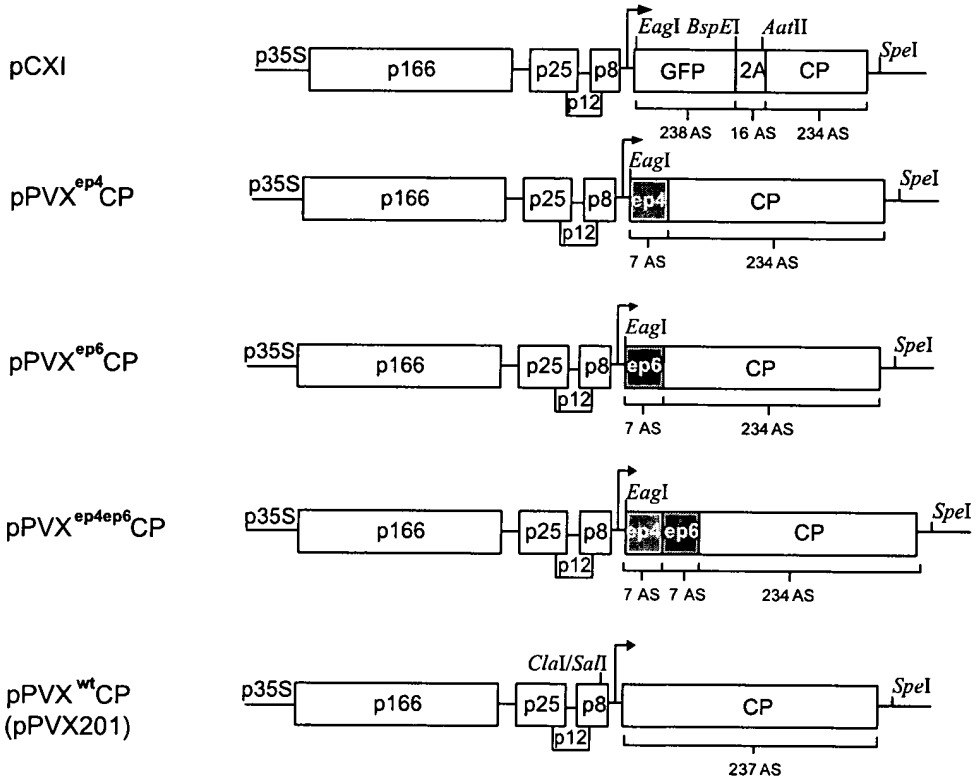

Fig. 1. Structure of PVX-derived vectors used for expression of wild type and recombinant coat proteins. Major features include the RNA-dependent RNA polymerase gene (166K), the triple gene block (25K, 12K, 8K), the virus coat protein gene (*CP*), the CaMV 35S promoter (*p35S*) and the additional BNYVV epitope sequences (*ep4* and *ep6*)

Table 1. Oligonucleotide primers used for preparative PCR and sequencing. *Eag*I restriction sites are underlined

Primer name	Nucleotide sequence (5′–3′)
CX-ep4	5′-AAACGGCCGATGAGTAGAACAAGCCCACCAGGACAACCCGCGAGCACA ACACAGC-3′
CX-ep6	5′-AAACGGCCGATGAGTAGCGCTAACGTCAGAAGAGACCCCGCGAGCACA ACACAGC-3′
CX-ep4ep6	5′-AAACGGCCGATGAGTAGAACAAGCCCACCAGGACAAAGCGCTAACGTCAGAA GAGACCCCGCGAGCACAACACAGC-3′
CX1	5′-TTGAAGAAGTCGAATGCAGC-3′
CX2	5′-CTAGATGCAGAAACCATAAG-3′
CX3	5′-ATAGCAGTCATTAGCACTTC-3′
CX4	5′-CGGGCTGTACTAAAGAAATC-3′

(Fig. 1). Plasmid DNA used for infection was amplified in the general *Escherichia coli* cloning strain DH5α.

Plant infection and PVX purification

Nicotiana benthamiana plants were inoculated with plasmid pPVX201 (a kind gift from D. Baulcombe, The Sainsbury Laboratory, Norwich, United Kingdom) which carries the wild-type PVX CP gene [3]. Further inoculations were carried out with plasmids $pPVX^{ep4}CP$, $pPVX^{ep6}CP$ and $pPVX^{ep4ep6}CP$, containing recombinant PVX genomes incorporating BNYVV epitopes (Fig. 1). Inoculation was achieved by gentle abrasion of the surfaces of three leaves per plant with carborundum and 5–10 μg of plasmid DNA. After inoculation, the surface of the leaves was rinsed with water in order to remove carborundum and excess DNA. Plants were maintained with a 16-h photoperiod (25,000–30,000 lux, 25 °C/20 °C temperature regime) and 60% humidity. Upon full systemic infection (about 14 days after inoculation) the correct expression of the foreign constructs was confirmed by enzyme-linked immunosorbent assay (ELISA), western blot and electron microscopy as discussed below.

PVX particles were purified according to a modified method of Koenig et al. [17]. Briefly, 50 g of leaf material stored at −80 °C was homogenized in two volumes of ice-cold 0.5 M boric acid solution (pH 7.8) in a Warring blender. After filtration through three layers of gauze, the pH was adjusted to 6.5 with HCl, and 0.2% (w/v) ascorbic acid and 0.2% (w/v) sodium sulfite were added. Cellular debris were removed by centrifugation (5500 × *g* for 20 min at 4 °C) and the supernatant was supplemented with 0.15 volumes of 0.5% silver nitrate and left at room temperature in the dark for 2–3 h. The solution was centrifuged as above and the second-stage supernatant was supplemented with one fifth of its volume of 1 M NaCl, 20% PEG in 0.5 M boric acid pH 7.8. Virus particles were sedimented by overnight incubation at 4 °C. The virus particles were pelleted by centrifugation (8000 × *g* for 30 min at 4 °C) and resuspended in 10 ml 0.5 M boric acid buffer (pH 7.8) containing 0.5 M urea and 0.1% β-mercaptoethanol. The solution was purified by centrifugation (8000 × *g* for 30 min at 4 °C) and the supernatant, containing the virus, was loaded onto a 30% sucrose cushion. After 150 min centrifugation at 72,500 × *g* (4 °C), the virus pellet was dissolved in 5 ml 0.5 M boric acid buffer (pH 7.8). The supernatant was clarified by centrifugation for 15 min at 6000 × *g* (4 °C), loaded onto a sucrose density gradient (10–45%, w/v in 0.5 M boric acid buffer pH 7.8) and centrifuged at 90,000 × *g* for 1 h at 4 °C using a Beckman SW28

rotor. Sucrose gradient fractions containing PVX particles, verified by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE), were combined and dialyzed against PBS (pH 7.4). The concentration of virus was calculated using the PVX extinction coefficient (2.97) for the extinction values at 260 nm.

Enzyme-linked immunosorbent assay (ELISA)

The presence of the foreign epitope on recombinant PVX particles in the sap of infected plants was confirmed using an enzyme-linked immunosorbent assay. Microtiter plates (Greiner, Germany) were coated for 2 h at 37 °C with 100 µl of anti-PVX polyclonal antibody (pAb) (DSMZ, Braunschweig, Germany) diluted 1:2000 in carbonate buffer (pH 9.6). After washing with phosphate buffered saline plus 0.05% (v/v) Tween-20 pH 7.4 (PBST), the plates were blocked with 2% skimmed milk powder in PBS for 1 h at 37 °C. Infected plant tissue was harvested and homogenized in PBS. Extracts were then added (100 µl per well) and plates were incubated at 37 °C for 2 h. After washing the plates, recombinant PVX particles were detected using monoclonal antibody (mAb) SCR84 or SCR86 (kindly provided by L. Torrance, SCRI, Dundee, United Kingdom) followed by incubation with an alkaline phosphatase (AP)-conjugated goat anti-mouse mAb (Dianova). Signal detection was achieved using *p*-nitrophenylphosphate (Sigma) as the substrate at a concentration of 1 mg ml^{-1} in substrate buffer (50 mM Tris-HCl, pH 9.6, containing 150 mM NaCl and 2 mM $MgCl_2$). The plate absorbance was read with an ELISA reader (Spectra Max 340) at 405 nm.

SDS-PAGE and western blot analysis

The protein profiles of crude sap extracts (1:3 dilution) or purified virus preparations, were analyzed by SDS-PAGE [22] using a 12% resolving gel and a 4% stacking gel, with samples incubated at 100 °C for 5 min in Laemmli loading buffer prior to loading. The separated proteins were transferred onto ImmobilonP nitrocellulose transfer membranes (Millipore) using the Mini Trans-Blot Cell (BioRad). Western blot analysis was performed using mAb SCR84, mAb SCR86 or pAb PVX as primary antibodies and AP-conjugated goat anti mouse or goat anti rabbit mAb (Dianova) as secondary antibodies.

Electron microscopy

Electron microscope grids with a Pioloform-carbon support film were floated for 5 min on drops of virus-infected plant sap, washed and blocked afterwards with 0.1% bovine serum albumin (BSA; Sigma) in phosphate buffer (PB, pH 7.2). Monoclonal antibodies recognizing BNYVV epitopes (SCR84, SCR86) were bound to the sample by incubating the grid in a PBST dilution of the appropriate antibody for 1.5 h. After washing (PBST), samples were decorated with gold-labeled goat anti-mouse IgG (15 nm, 10 nm or 5 nm gold particles, Biocell, United Kingdom) by floating the grid on a 1:50 PBST dilution of the antibody for 2 h or overnight. The grids were extensively washed (once with PBST, and twice with distilled H_2O) and the sample stained with five drops of 1% uranyl acetate. Electron microscopy was carried out using a Zeiss 906 transmission electron microscope.

Sequencing

Sequencing was performed using an ABI Prism 3700 DNA Analyzer (Applied Biosystems), primers CX2 and CX4 (Table 1), and the ABI Prism BigDye Terminator v3.0 Ready Reaction Cycle Sequencing Kit (Applied Biosystems).

Results

Production and detection of hybrid viruses

Chimeric CP molecules were produced by adding nucleotide sequences encoding different BNYVV epitopes to the 5′-end of the PVX coat protein gene, resulting in the three recombinant PVX constructs shown in Fig. 1 ($PVX^{ep4}CP$, $PVX^{ep6}CP$, $PVX^{ep4ep6}CP$). Plasmid DNA containing the full-length wild type cDNA of PVX (pPVX201; Fig. 1) or PVX with modified CP sequences ($PVX^{ep4}CP$, $PVX^{ep6}CP$, $PVX^{ep4ep6}CP$; Fig. 1) was used to inoculate *N. benthamiana* plants. One to two weeks post inculation (p.i.), all plants showed systemic infections and symptoms identical to those of plants infected with wild-type PVX (Fig. 2). Systemically infected leaves were collected, and the expression of foreign sequences was verified by ELISA (data not shown) and western blot (Fig. 3).

Virus particles were purified from the systemically infected leaves and yields of up to 115 μg per gram of leaves were achieved (data not shown). Total protein extracts from infected leaves as well as purified virus preparations were separated by SDS-PAGE. As shown in Fig. 3, the wild type CP and the three recombinant

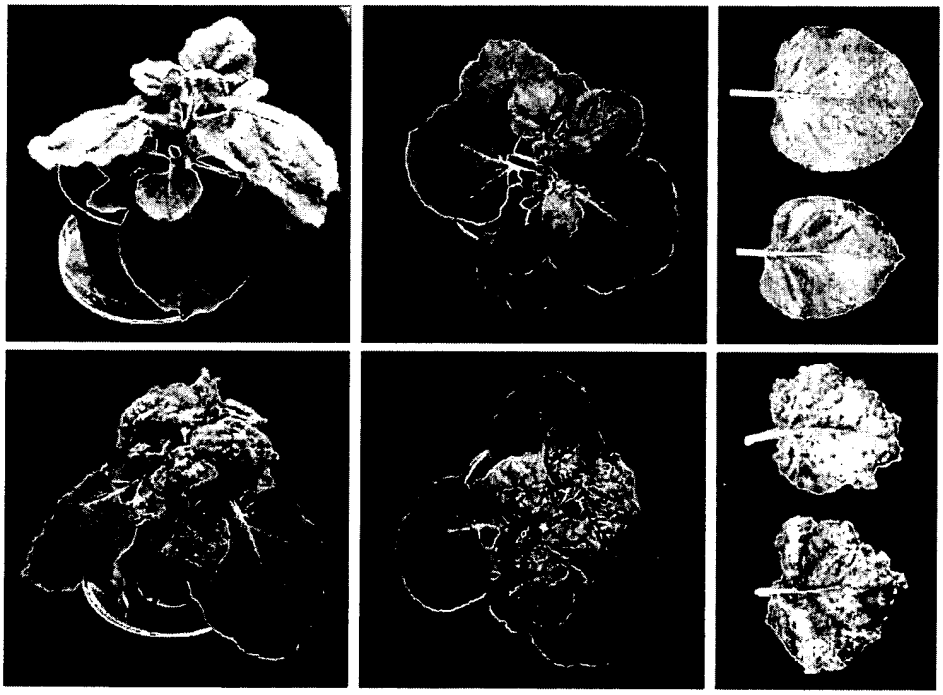

Fig. 2. PVX infection of *N. benthamiana* plants, showing whole plants viewed from the side and the top, and two sample leaves. Top row: Non-infected control plant. Bottom row: Infected plant 14 days after inoculation of three leaves with $pPVX^{ep6}CP$ (5–10 μg per leaf)

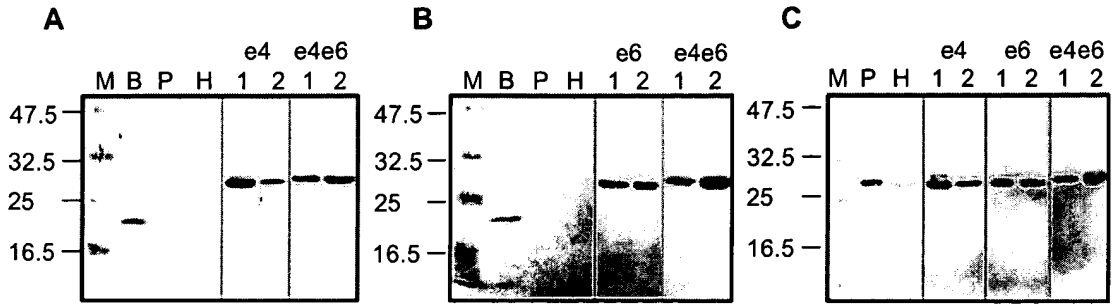

Fig. 3. Western blot analysis of $PVX^{wt}CP$ and $PVX^{epX}CP$ infected *N. benthamiana* plant extracts (24 days p.i.) diluted 1:3 in PBS. Antibody detection: **A**: mAb SCR86 + goat anti-mouse-alkaline phosphatase (GAM^{AP}). **B**: mAb SCR84 + GAM^{AP}. **C**: pAb PVX + goat anti-rabbit-alkaline phosphatase (GAR^{AP}). Lane designations: *M* = prestained broad range marker (New England Biolabs); *B* = BNYVV infected *Chenopodium quinoa* plant sap; *H* = non-infected control plant; *P* = $PVX^{wt}CP$ control; *e4* = $PVX^{ep4}CP$; *e6* = $PVX^{ep6}CP$; *e4e6* = $PVX^{ep4p6}CP$; *1*, *2*: two different $PVX^{epx}CP$ infected plants

CPs migrated close to their calculated positions with molecular masses of 25.05, 25.77, 25.85 and 26.57 kDa, respectively.

Immunogold labeling of hybrid PVX particles

Immunogold electron microscopy using monoclonal antibodies specific for each of the two BNYVV epitopes revealed binding to the surface of purified PVX particles as shown in Fig. 4A–D. This confirmed that the foreign epitopes were accessible and exposed on the surface of the PVX virions. Furthermore, viruses expressing both epitopes in a tandem array construct were detected by both antibodies either singly or in combination (Fig. 5A–D). This showed that the tandemly arranged epitopes were individually accessible on the surface of the particles, and could also be accessed simultaneously by different antibodies labeled with different-sized gold particles (Fig. 5C and D). Double labeling of the $PVX^{ep4ep6}CP$ particles was accomplished by incubation with the primary ep6-specific mAb and a 5 nm gold-labeled secondary mAb followed by incubation with a mixture of the primary ep4-specific mAb and a 10 nm-gold labeled secondary mAb. The possibility that double labeling with different-sized gold grains was obtained by exchange of secondary antibodies was excluded by the results of a mixed infection described in the following section.

PVX infection with a mixture of two different DNA constructs

To establish the number of epitopes presented in mixed infections, *N. benthamiana* plants were inoculated with a 1:1 mixture of constructs $pPVX^{ep4}CP$ and $pPVX^{ep6}CP$. In such infections, ^{ep6}CP and ^{ep4}CP could be synthesized in the same cell and virus particles could be formed with both epitopes presented on

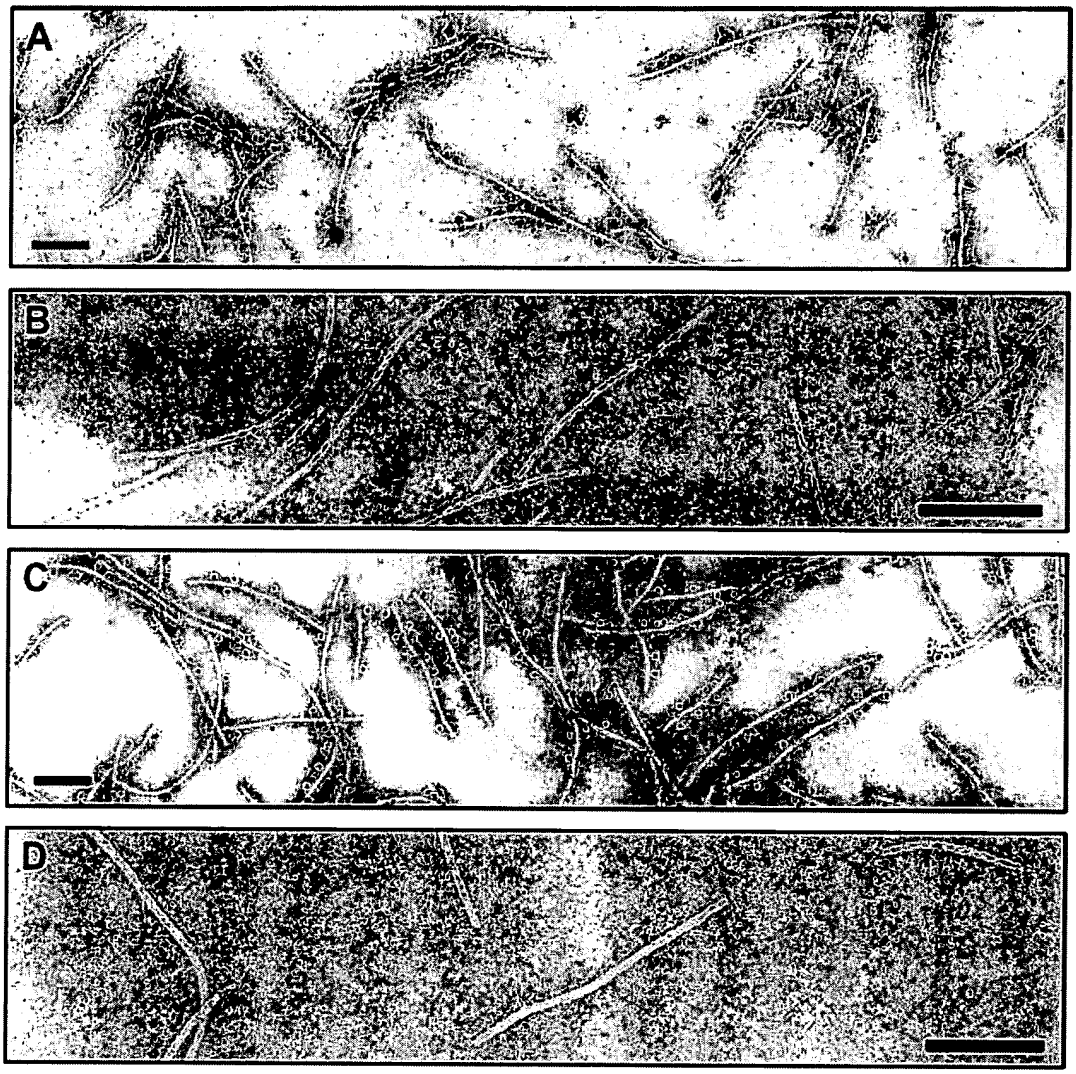

Fig. 4. Immunogold labeling of $PVX^{ep}CP$ particles from infected *N. benthamiana* plants **A**: $PVX^{ep4}CP$ decorated with mAb SCR86 specific for BNYVV epitope 4 (1:5 dilution in PBST), detection with 15 nm goat anti-rabbit (GAR) gold conjugate (1:50 dilution in PBST). **B**: Negative control, $PVX^{ep4}CP$ particles, primary mAb SCR84 specific for BNYVV epitope 6 (1:10 dilution in PBST), secondary pAb 15 nm GAR gold conjugate (1:50 dilution in PBST). **C**: $PVX^{ep6}CP$ particles decorated with mAb SCR84 specific for BNYVV epitope 6 (1:10 dilution in PBST), detection with 15 nm gold conjugate GAR (1:50 dilution in PBST). **D**: Negative control, $PVX^{ep6}CP$ particle, primary mAb 3H12 specific for BNYVV epitope 4 (1:10 dilution in PBST), secondary pAb 15 nm gold conjugate GAR (1:50 dilution in PBST). Bar = 200 nm

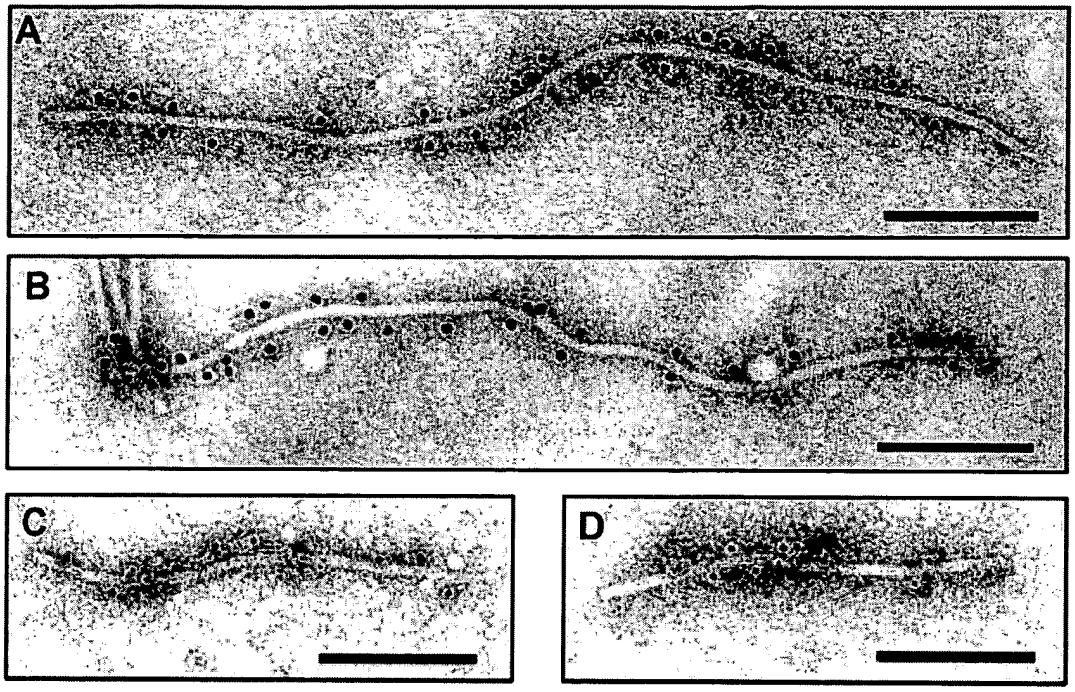

Fig. 5. Immunogold labeling of $PVX^{ep4p6}CP$-particles (tandem epitope) from *N. benthamiana*. **A**: $PVX^{ep4p6}CP$-particles decorated with mAb SCR86 specific for BNYVV epitope 4 (1:5 dilution in PBST), detection with goat anti-rabbit (GAR) 15 nm gold conjugate (1:50 dilution in PBST). **B**: $PVX^{ep4p6}CP$ particles decorated with mAb SCR84 specific for BNYVV epitope 6 (1:10 dilution in PBST), detection with GAR 15 nm gold conjugate (1:50 dilution in PBST). **C** and **D**: $PVX^{ep4p6}CP$ particles decorated with mAb SCR84 (1:10 dilution in PBST) and goat anti-mouse (GAM) 5 nm gold conjugate (1:50 dilution in PBST) and mAb SCR86 (1:5 dilution in PBST) saturated with GAM 10 nm gold (1:50 dilution in PBST). Bar = 200 nm

the surface. Purified PVX particles from mixed infections were initially incubated with the primary mAb specific for one of the two epitopes, and binding was detected with a 15 nm gold-labeled secondary antibody. This analysis showed that such preparations contained both gold-labeled virions and a fraction of completely undecorated virions (Fig. 6A and B). Double labeling was then carried out as described in the previous section. Incubation with the primary ep6-specific mAb and a 5 nm gold-labeled secondary mAb was followed by incubation with a mixture of the primary ep4-specific mAb and a 10 nm-gold labeled secondary mAb. As shown in Fig. 6D, PVX virions were identified that were labeled either with 5 or with 10 nm gold particles alone, but there were also virions that were labeled simultaneously with gold particles of both sizes (Fig. 6C and E).

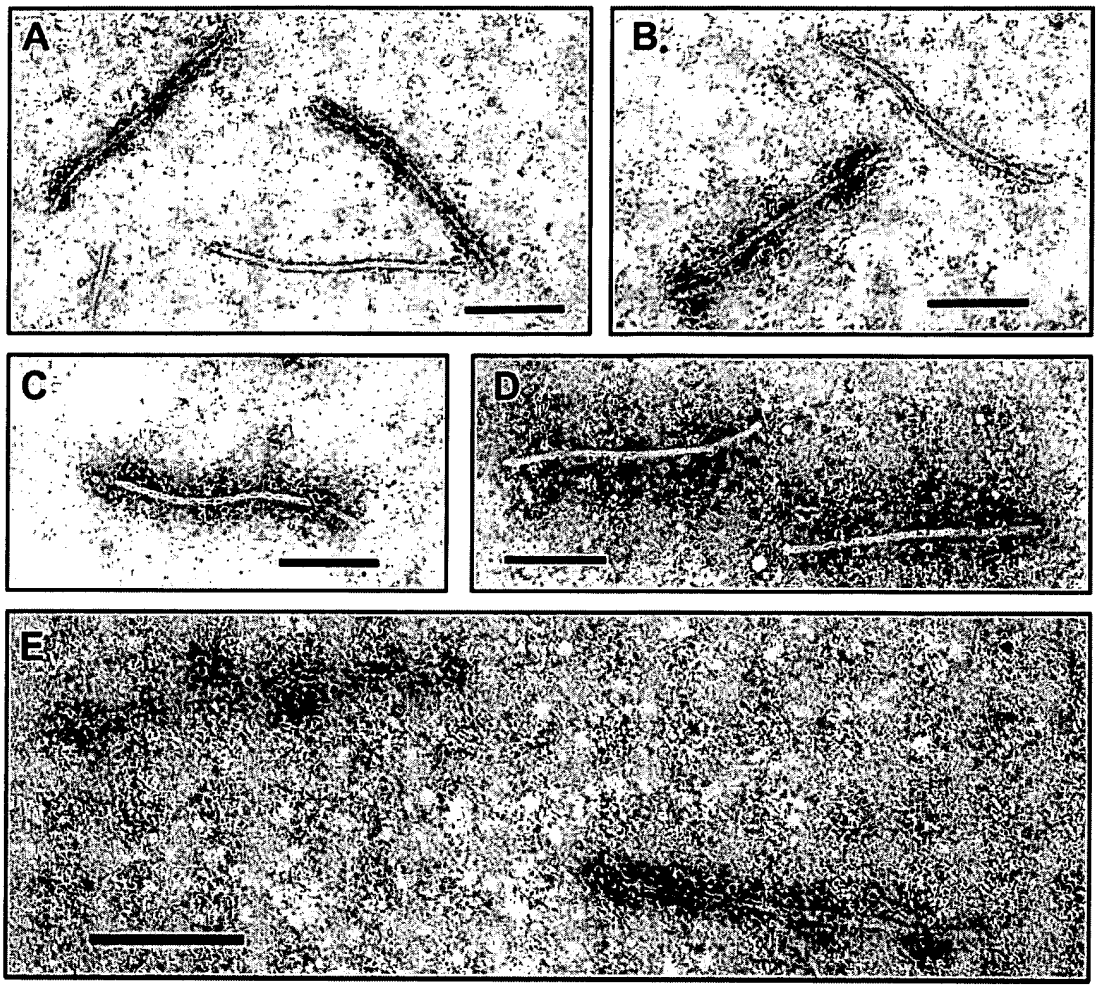

Fig. 6. Immunogold labeling of PVX^{ep4}CP and PVX^{ep6}CP particles from mixed infections. **A**: PVX^{ep4}CP + ^{ep6}CP particles decorated with mAb SCR86 specific for BNYVV epitope 4 (1:5 dilution in PBST) detected with goat anti-rabbit (GAR) 15 nm gold conjugate (1:50 dilution in PBST). **B**: PVX^{ep4}CP + ^{ep6}CP-particles decorated with mAb SCR84 against BNYVV epitope 6 (1:10 dilution in PBST), detected with GAR 15 nm gold conjugate (1:50 dilution in PBST). **C–E**: PVX^{ep4}CP + ^{ep6}CP-particles decorated first with mAb SCR84 (1:60 dilution in PBST) and goat anti-mouse (GAM) 5 nm gold conjugate (1:50 dilution in PBST) and then with mAb SCR86 (1:5 dilution in PBST) saturated with GAM 10 nm gold conjugate (1:50 dilution in PBST). Bar = 200 nm

Discussion

The use of plants as bioreactors for the synthesis of proteins has the potential to reduce the cost of protein therapeutics, and is especially useful for vaccine production and delivery [36]. Vaccines can be expressed as full length polypeptides

in transgenic plants, but epitope presentation on the surface of virus particles is a useful alternative which is quicker and does not require genetic transformation [23]. PVX is a good candidate carrier molecule for foreign epitopes because it self-assembles in an ordered manner, and accumulates to high levels in infected tissues. It also lacks the size and packaging constraints of icosahedral viruses like CPMV.

In this study we have used PVX vectors to express BNYVV epitopes fused to the N-terminus of the PVX coat protein. The seven-amino-acid epitopes ep4 and ep6 [39], as well as the 14-amino-acid tandem epitope ep4ep6, were expressed successfully in systemically infected *N. benthamiana* plants and were exposed on the surface of the recombinant PVX particles. Therefore, these additional sequences appear not to interfere with virus assembly nor with cell-to-cell movement, even though the PVX CP is known to be required for systemic spread during natural infections [34].

The assembly of recombinant PVX particles containing a six-amino-acid HIV epitope fused to the N-terminus of the CP has been reported [24]. To the best of our knowledge, however, we are the first to demonstrate the assembly of virions displaying tandem epitopes and the first to demonstrate the display of multiple epitopes on single virus particles following mixed infections. In each case, we have shown that both epitopes can be accessed and recognized by their cognate antibodies either individually or simultaneously, suggesting that either strategy could be useful for the simultaneous presentation of multiple antigens in vaccination programs. While recombinant viruses expressing the tandem construct display both epitopes, mixed infections result in the production of virions displaying ep4 alone, ep6 alone or both epitopes (Fig. 6).

The first experiments with PVX CP fusion proteins established that PVX assembly is blocked by the presence of foreign proteins [9]. For this reason, the FMDV 2A peptide [29] has been inserted between the heterologous sequence and the CP open reading frame in previous studies to allow a degree of co-translational cleavage and the production of some wild type CP subunits [15, 30]. Recently, it has been shown that this process is not proteolytic, but instead involves ribosomal skipping on the RNA template [11, 12]. Consequently the production of recombinant and wild type coat protein is accomplished, infectious particles are formed and proteins of 8.5 to 46 kD can be presented on the virus surface [9, 25, 32]. One disadvantage of this "overcoat" principle, however, is the unpredictable ratio of wild type to recombinant CP, which may vary between 1:1 and 1:5 [21, 25]. If peptide-presenting viruses are to be used as standards in diagnosis or vaccination, the presentation of epitopes on all CP subunits is advantageous, since this assures an exactly defined and high concentration of desired antigenic sites.

Our ultimate goal is to produce protein standards presenting a) several different epitopes concurrently or b) several short epitopes, either combining epitopes from different viruses in one standard or using different epitopes from the same virus to design a standard recognized by many different antibodies available against this virus. The latter would be particularly advantageous for simplification and reduction of standard production costs. Furthermore, the presentation of several epitopes on plant virions would also be very interesting for vaccine development,

since antibodies that are produced against a single pathogen epitope are often not sufficient to provide complete and long lasting protection against an infection [1, 20, 31].

The size of the longest peptide presented on TMV virions was 23 residues [5] and on PPV and CPMV particles up to 30 and 50 residues, respectively [13, 26, 28]. Although this capacity would potentially allow for the presentation of several shorter epitopes, there have been only few reports about the presentation of multiple peptides on these and other plant virions [13, 14, 38]. None of these studies addressed the accessibility of the individual epitopes, instead their efficiency to elicit protective antibodies was investigated. The construction of a CPMV chimera with an insert of *Pseudomonas* epitopes 18 and 10 in tandem within the L or S subunit of the virus elicited antibodies that were directed only toward the epitope 10 or 18, respectively [14]. Obviously the CPMV CP subunit into which the peptide was inserted greatly influenced the nature of the antibodies elicited [6]. PPV chimeric virions presenting different forms of an antigenic peptide (single and tandem repetition) from the VP2 capsid protein of canine parvorvirus (CPV) showed a high antigenicity [13]. However no substantial difference was observed in the quality of immune response induced by single or tandem presentation of the CPV peptide.

The expression of peptides as coat protein fusions can lead to the production of large amounts of antigen. However, in order to prevent any undesirable changes in the aa sequence of the epitope through mutations, the recombinant particles should be isolated from infected plants after a relatively short period of time (2–3 weeks). This helps to prevent the accumulation of mutated viruses in which the epitope has been altered or even deleted, as observed after serial passages in *N. benthamiana* (Uhde K. & Commandeur U., unpublished results). Therefore, it is important to verify the presence and accessibility of foreign epitope sequences using appropriate antibodies.

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